Exhibit A

Gene Therapy (2001) 8, 704–712

www.nature.com/gt

RESEARCH ARTICLE

Recombinant adenovirus expressing adeno-associated virus cap and rep proteins supports production of high-titer recombinant adeno-associated virus

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It has been difficult to produce a chimeric vector containing both Ad and AAV rep and cap, and to grow such chimeric vectors in 293 cells. By recombination in vitro in a bacterial host, we were able to produce recombinant plasmid AdAAV (pAdAAVrep-cap), which could be used to generate recombinant AdAAV (rAdAAVrep-cap) after transfection into 293 cells. A recombinant adenovirus, rAdAAVGFP, in which the green fluorescent protein (GFP) gene is flanked by the AAV terminal repeats cloned into the E1-deleted site of Ad was also generated. Co-infection of rAdAAVrep-cap together with rAdAAVGFP into 293 cells resulted in production of high titers of rAAV expressing GFP. It was noted that the titer of rAdAAVrep-cap was lower than the titer of control AdCMVLacZ. The lower titer of rAdAAvrep-cap was associated with expression of Rep protein. Non-homologous recombination occurs after high passage and results in deletions within the AAV rep genes. These results indicate that (1) rAdAAVrep-cap can be produced; (2) rAdAAVrep-cap + rAdAAVGFP is a convenient and efficient way to transfect 293 cells to grow high titer rAAV; and (3) frozen stock is required to avoid propagation of rep-deleted pAdAAVrep-cap. Gene Therapy (2001) **8,** 704–712.

Keywords: *adenovirus; adeno-associated virus; chimeric vector; AdAAV; rep; cap*

Introduction

Successful gene therapy requires the development of vectors that can sustain long-term expression of the gene in the host without incurring a pathogenic response. Adeno-associated virus (AAV) is not pathogenic in humans and it is hoped that the ability of AAV to undergo site-specific integration *in vivo* can be exploited to prolong the life of the vector and the expression of the therapeutic gene.[1,2] Its utility as a vector has been limited, however, by the lack of a strategy for large scale and high-titer production of the recombinant AAV. In addition, the AAV genome size limitation of 4.68 kb prevents large cDNA insertion although recent results indicated that this size limitation can be increased by intermolecular joining of two complementary vectors.[3–5]

AAV utilizes two mechanisms to replicate. In the absence of helper virus, the AAV viral genome can become integrated in a site-specific manner into the host cell genome. Replication of AAV also can occur after subsequent infection with a helper virus, typically either adenovirus (Ad) or herpesvirus.[6–8] The current method of production of rAAV requires transfection of multiple DNA vectors into either a packaging cell line or 293 cells.[9–12] The necessary helper functions are then provided by infection with a helper adenovirus (Ad) or transfection with plasmid DNA encoding Ad helper functions.[9] Although several packaging cell lines expressing the rep and cap gene products have been successfully used to achieve relatively high-titer production of rAAV, large-scale production of rAAV is still limited by the requirement for transfection of multiple DNAs encoding Ad helper functions or AAV rep and cap gene products.[9] It would be desirable to generate a hybrid adenovirus expressing the AAV rep and cap gene products (rAdAAVrep-cap) to achieve large-scale and high-titer production of the recombinant AAV. Efficient production of recombinant AAV could be achieved through a strategy utilizing two recombinant helper viruses in which one recombinant helper virus contains the AAV rep and cap genes and the second contains the therapeutic gene flanked by the AAV packaging and replication signals that are contained in the ITRs. This strategy would also facilitate site-specific integration of recombinant AAV by co-transfection of the recombinant helper virus containing the AAV genetic components and site-specific integration could be achieved by AAV Rep-mediated site-specific integration. Previous investigators have failed, however, to generate hybrid rAdAAV by direct DNA homologous recombination in 293 cells. This was proposed to be due to an inhibition of adenovirus replication by the AAV reps, although the mechanism underlying the inhibition has not been established.[13,14]

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Received 26 June 2000; accepted 7 February 2001

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Four Rep proteins are encoded within a single open reading frame of AAV. These four Rep proteins include Rep78, Rep68, Rep52, and Rep40, which are produced by alternative RNA splicing of RNA products that are transcribed from two different translation codons.[2,6] Rep78 and Rep68, which are produced from the same p5 promoter have been proposed to be toxic.[13,14] These two proteins, which are essential not only for site-specific integration but also for AAV replication, have several biochemical activities in common and both interact with the DNA-specific binding site of Rep.[15] Both Rep78 and Rep68 exhibit strand- and site-specific endonuclease activity,[16] as well as an ATP-dependent DNA-DNA and DNA-RNA helicase activity.[17] Both are able to modulate the activity of endogenous as well as heterologous promoters.[18–20] They have been reported to down-regulate the expression of human genes such as c-H-ras, c-fos, c-myc, and c-sis[21–24] and can inhibit the proliferation of some cell lines[25,26] and lead to p53-mediated apoptosis. Due to these complex and multiple functions of Rep78 and Rep68, it has been difficult to produce chimeric viruses or stable cell lines that express these proteins.

Recchia *et al*[27] have recently generated Ad/AAV expressing Rep 78 driven by the bacterial phage T7 promoter to minimize Rep expression and has shown this facilitates site-specific integration of an AAV-ITR-flanked DNA. Hybrid HSVAAV has also been produced by construction of a hybrid HSV/AAV amplicon plasmid or ICP27 deleted HSV/AAV.[28,29] The recombinant hybrid HSVAAV genome could be packaged into the HSV viral particle, and support recombinant AAV production as a helper virus. We speculate that the failure to make recombinant AdAAV may be partially due to AAV Rep interference with Ad genomic DNA homologous recombination *in vivo*. To avoid the unidentified factors that may affect generation of AdAAV *in vivo*, we developed a strategy for producing recombinant plasmid pAdAAV *in vitro*. Recombinant pAdAAV with the rep and cap genes (rAdAAVrep-cap) was generated by homologous DNA recombination in a bacterial host. We also produced a recombinant pAdAAV that contained the GFP flanked by the AAV-ITR (pAdAAVGFP). The hybrid plasmids were then used to successfully generate recombinant adenovirus (rAdAAVrep-cap and rAdAAVGFP), respectively. Next, we demonstrated that co-transfection of both rAdAAVrep-cap and rAdAAVGFP can produce high-titer recombinant rAAV that expresses the marker gene GFP in 293 cells. Finally we observed that at high passage number, AAV reps were deleted from the rAdAAVrep-cap, and a non-homologous deletion specifically occurred within the rep gene of rAdAAV-rep-cap.

Results

Construction of the recombinant rAdAAVrep-cap

*Pac*I linearized pAdAAVrep-cap (Figure 1a) was transfected into 293 cells using the LipofectAmine procedure. Visible viral plaques had developed 12 days after transfection in contrast to 7 days when *Pac*I linearized pAdeasy1 was used as a control. These were harvested and the positive clones were identified and verified by restriction enzyme digestion with *Xba*I, *Sal*I and *Eco*RI (Figure 1b). The restriction enzyme products mapped as expected for

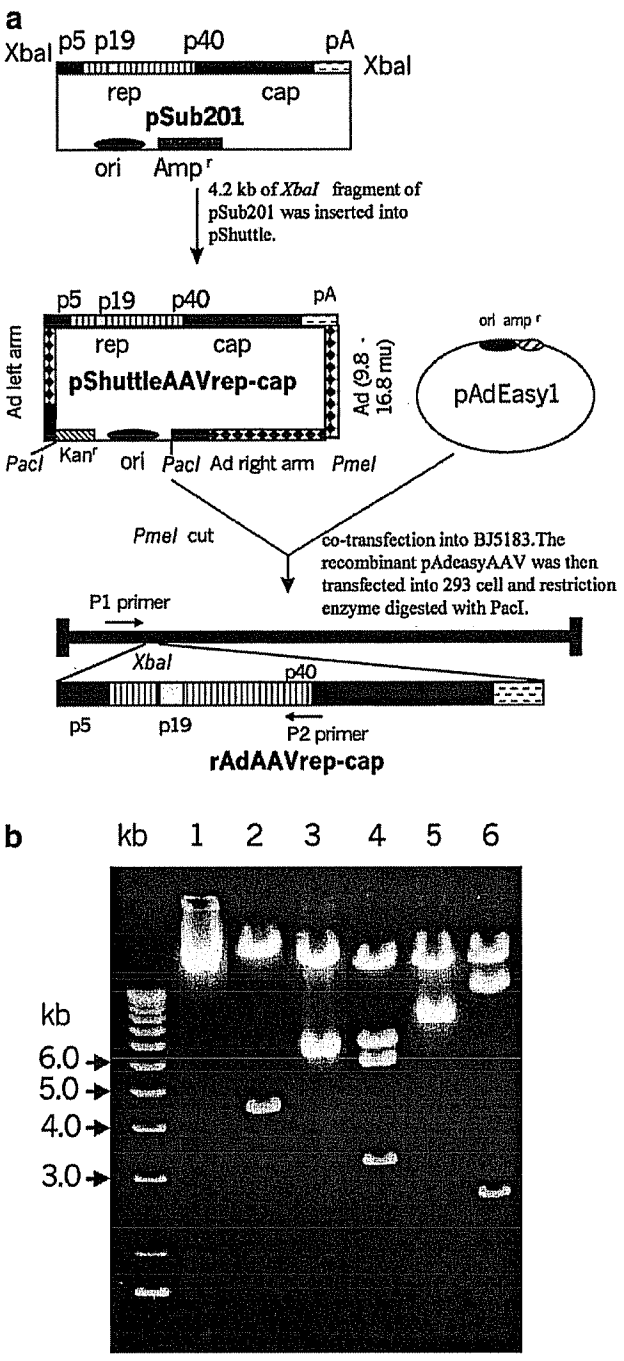

Figure 1 Construction of hybrid rAdAAVrep-cap. (a) Diagrammatic representation of the construction strategy. The XbaI fragment encoding AAV rep and cap genes was excised from the AAV pSub201, and inserted into the XbaI site of pShuttle. After linearization with PmeI, 1 μg of pShuttleAAVrep-cap was mixed with pAdeasy1, and co-transformed into BJ5183 cells. The correct recombinant pAdAAVrep-cap was identified by restriction enzyme digestion. The P5 promoter drives rep78 and rep68 genes and the p19 promoter drives rep52 and rep40 genes. The P40 promoter drives expression of the cap gene. P1 and P2 primers indicate the location of PCR primers for analysis of rep deletion. (b) XbaI, SalI and EcoRI restriction maps of recombinant rAdAAVrep-cap DNA. 20 μg of rAdAAVrep-cap genomic DNA were cut with XbaI, SalI, and EcoRI, respectively. After digestion 1 h at 37°C, the samples were electrophoresed on a 1% agarose gel and photographed under UV light, the far left-hand lane is a 1 kb ladder (BRL). Lane one, XbaI-digested AdEasy1; lane 2, XbaI-digested rAdAAVrep-cap; lane 3, SalI-digested AdEasy1; lane 4, SalI-digested rAdAAVrep-cap; lane 5, EcoRI-digested AdEasy1; lane 6, EcoRI-digested rAdAAVrep-cap.

the proper recombinant plasmid (Figure 1b). Digestion of rAdAAVrep-cap with *Xba*I revealed the 4.3 kb AAV fragment containing the cap and rep genes. *Sal*I digestion of rAdAAVrep-cap revealed one unique 3.3 kb fragment representing the unique *Sal*I site of AAVrep-cap. *Eco*RI-digested rAdAAVrep-cap revealed one additional 2.4 kb fragment representing the integration of the AAV *Eco*RI site located in the rep gene into the rAdAAVrep-cap genomic DNA. The restricted mapped rAdAAVrep-cap viral genomic DNA was further verified by partial sequencing of the PCR amplified insert using primers flanking the E1 deleted region (data not shown).

rAdAAVrep-cap can express AAV rep and cap mRNA

To determine if AAV rep and cap genes were expressed after transfection with recombinant rAdAAVrep-cap, the 293 cells were transfected with rAdAAVrep-cap at 10 p.f.u./cell and total RNA was extracted at 16 h after infection using the TriZol extraction method. The production of AAV rep and cap mRNA was analyzed by Northern blot analysis. The blot was hybridized with an [α-^{32}P]-dCTP labeled AAV fragment (*Xho*I and *Xba*I fragment of AAV genomic DNA encoding AAV partial rep and cap genes from pSub201). All AAV transcripts were detected at 16 h after infection. Hybridization bands were observed at approximately 2.3 and 2.6 kb, which correspond to the cap mRNA species, as well as bands at 3.3, 3.6, 3.9 and 4.2 kb were observed, which correspond to the rep RNA species (Figure 2a). Production of AAV cap protein requires functionally intact cap and rep genes. The presence of cap proteins was determined by analysis of 50 μg of total protein isolated from rAdAAVrep-cap-infected 293 cells fractionated by 10% polyacrylamide-electrophoresis. Immunoblot analysis using AAV capsid probes demonstrated that all three AAV capsids were translated successfully and were not present in the control AdCMVLacZ infected cells (Figure 2b).

Efficient excision and replication of rAAVGFP

To determine if co-transfection of rAdAAVrep-cap and rAdAAVGFP can lead to replicating rAAVGFP, the Hirt extracted low molecular weight DNA was analyzed by Southern blot using a GFP probe (Figure 3). The results showed that three genomic forms were generated during the replication process: an approximate 4 kb corresponding to monomers, an approximate 8 kb corresponding to dimers, and the high-molecular-weight form corresponding to full-length rAdAAVGFP viral genomic DNA. The rAAVGFP viral genomic DNA replicated more efficient at 40 h compared to 24 h after co-infection of 293 cells with the rAdAAVrep-cap and rAdAAVGFP hybrid virus (lanes 2 and 3). In addition, neither DNAs extracted from non-infected 293 cells nor AdCMVLacZ infected 293 cells exhibited the monomer or dimer-replicative DNA (lanes 1 and 4).

Expression of AAV genes in an rAdAAVrep-cap hybrid vector inhibits the replication of recombinant Ad

To determine if rAdAAVrep-cap could be grown successfully in 293 cells, the titer of rAdAAVrep-cap was determined at different times after infection. Similar infection of the cells with AdCMVLacZ (3.8×10^3 p.f.u./cell) resulted in virus production that peaked 40 h after infection (Figure 4a). Infection with rAdAAVrep-cap resulted in inhibition of Ad5. The titer of rAdAAVrep-cap (2.4×10^2 p.f.u./cell) significantly differ from that of control AdCMVLacZ ($P < 0.05$). These results indicate that AAV Rep inhibits adenovirus replication. To determine if inhibition of Ad replication is AAVRep-specific, *Pac*I linearized pAdeasy1 was co-transfected with increasing amounts of a Rep expression vector, pShuttleAAVrep-cap, as shown in Figure 1a. The production of Ad5 was found to be delayed in the presence of Rep in a dose-dependent fashion (Figure 4b). Moreover, recombinant rAdAAVrep-cap was not identified by PCR screening. Thus, the delay in Ad growth in the presence of rAdAAVrep-cap may be due to the rep gene product.

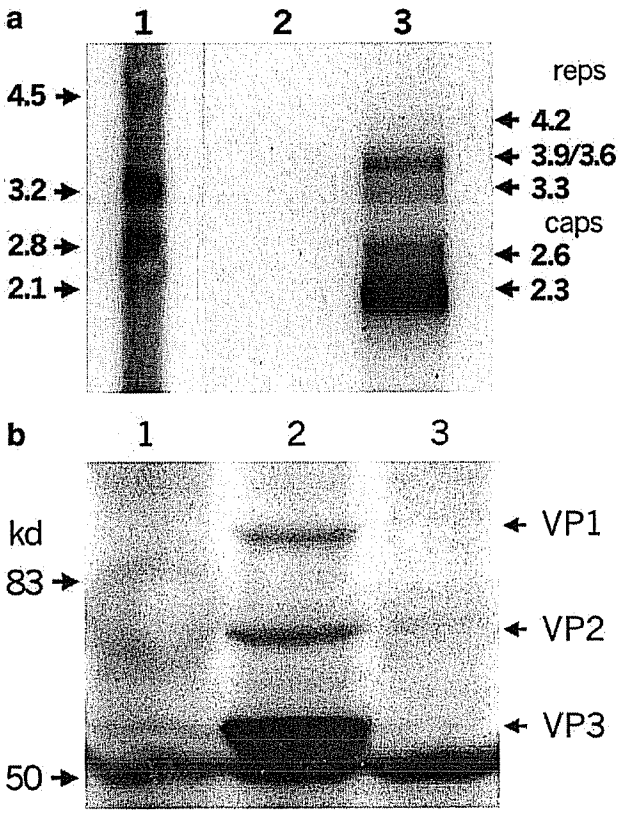

Figure 2 A hybrid rAdAAVrep-cap can express AAV rep and cap genes. (a) Northern blot analysis of AAV mRNAs. 10 μg of total RNA extracted from rAdAAVrep-cap infected 293 cells was transferred on to a nylon membrane, and hybridized with [α-^{32}P]-labeled AAV probe (nt position from 2202 to 4344 bp, XhoI and XbaI fragment of AAV genome). The film was exposed for 2 h. Lane 1, RNA marker (BRL); lane 2, total RNA extracted from 293 cells infected with AdCMVLacZ (5 p.f.u./cell); lane 3, total RNA extracted from 293 cells infected with rAdAAVrep-cap (5 p.f.u./cell). (b) Immunoblot analysis of AAV capsids. Total protein extracted from 293 cells treated as described in Materials and methods was blotted with anti-AAV capsid protein (B1 clone, American Research Production, Belmont, MA, USA), and signal was detected using the ECL system. Lane 1, protein extracted from 293 cells transfected with AdCMV-LacZ (5 p.f.u./cell); lane 2, protein extracted from 293 cells transfected with rAdAAVrep-cap (5 p.f.u./cell); and lane 3, protein extracted from uninfected 293 cells.

Production of rAAVGFP by co-infection of rAdAAVrep-cap and rAdAAVGFP

rAdAAVrep-cap and rAdAAVGFP were co-infected with an equal concentration of 5 p.f.u./cell into 293 cells. This led to lysis of 80% of the cells at 40 h after infection. The

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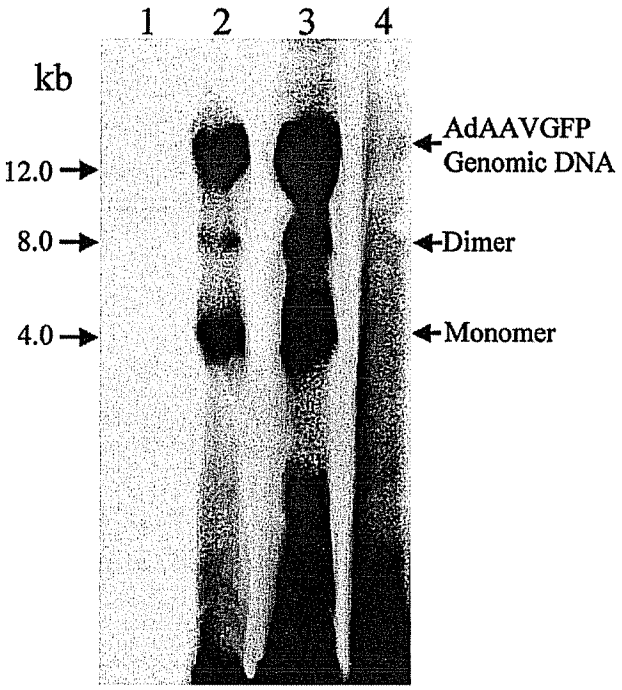

Figure 3 Efficient replication of recombinant AAVGFP after co-infection of rAdAAVrep-cap and rAdAAVGFP in 293 cells. 293 cells were co-infected with rAdAAVrep-cap and rAdAAVGFP at 10 p.f.u./cell, low molecular weight DNAs were extracted at 24 h (lane 2), or 40 h (lane 3) after infection using Hirt extraction method as described in method section. A ^{32}P-dCTP labeled GFP probe was used for detection of replicative forms of the recombinant AAVGFP including monomer (approximately 4.0 kbp) and dimer (approximately 8.0 kb). In addition, non-infected 293 cells (lane 1) or 293 cells infected with AdCMVLacZ (lane 4) were used. The signals were developed by exposure the membrane to X-ray film, and the film was developed 3 h after exposure.

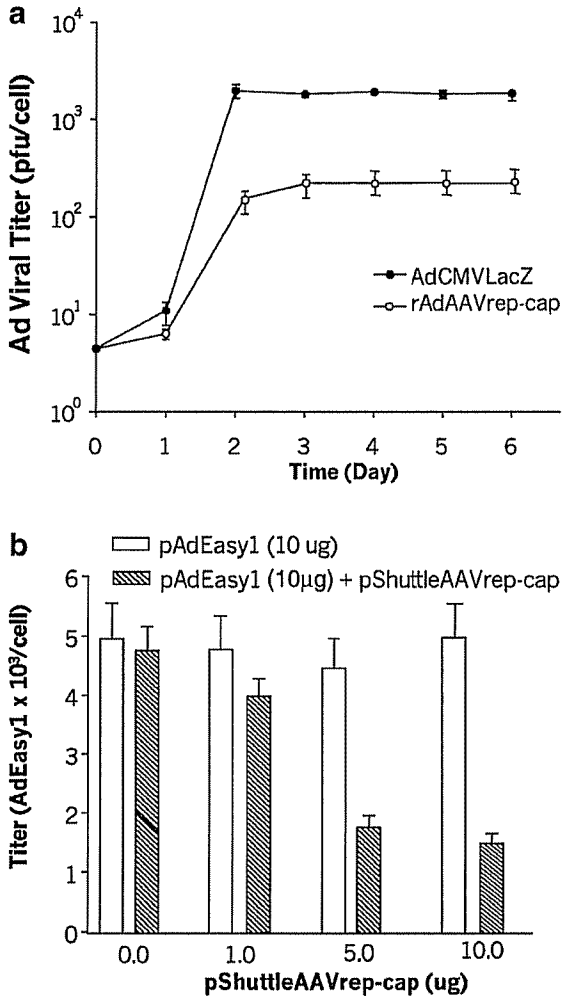

Figure 4 A hybrid rAdAAVrep-cap virus inhibits Ad replication. CsCl-purified rAdAAVrep-cap or AdCMVlacZ was used to infect 293 cells at dose of 10 p.f.u./cell. The cells were infected in triplicates in 24-well plates. The cell pellets were collected as indicated. The pellets were frozen and thawed three times before titration. The virus was titrated by 10-fold dilutions of harvested stock. The viral titer was read at 48 h after infection, and expressed as plaque forming units/cell. (b) Dose-dependent delayed production of Ad by pShuttleAAVrep-cap. PacI-linearized pAdeasy1 was co-transfected with elevated doses of pshuttleAAVrep-cap expressing rep and cap into 293 cells using the LipofectAmine method (BRL). When the viral plaques were observed, the total infected cells were lysed by thaw and frozen three times. The viral titer was determined by limited dilution plaque assay. The possibility of production of recombinant rAdAAVrep-cap was screened by PCR amplifying rep genes using primers as described in Figure 5.

data indicate that a very high titer rAAVGFP (0.48×10^3 TU/cell) can be produced using this procedure (Figure 5). The titer of rAAVGFP was approximately six-fold higher than the titers achievable using transfection of pShuttleAAVGFP DNA into the AAV packaging cell line B50 followed by Ad309 infection.

Stability of rAdAAVrep-cap after passage in 293 cells

After rAdAAVrep-cap had undergone between eight and 16 passages through 293 cells, we found that rAdAAV-rep-cap lost its ability to support production of rAAVGFP. To determine the basis for this loss in its ability to support production, the rAdAAVrep-cap vector DNA was sequenced after passage through 293 cells for four passages, eight passages and 16 passages. Using specific primers, the genomic integrity of the AAV insert was examined after different numbers of passages (Figure 6a). The primer sequences were designed to probe the adenovirus vector sequences that flank the incorporated AAV in the rAdAAVrep-cap virus, specifically those sequences flanking the rep genes. After four passages, the full-length (2.2 kb) rep genes were incorporated into the rAdAAVrep-cap chimeric vector (Figure 6a, lane 2), and were identical in size to the wild-type rep genes (Figure 6a, lanes 5 and 6). After eight passages, there was an appearance of an approximately 0.6 kb rep gene incorporated into the rAdAAVrep-cap chimeric vector (Figure 6a, lane 3), however the majority of rAdAAVrep-cap contained a full-length 2.2 kb fragment. After 16 passages (Figure 6a, lane 4), most of the rAdAAVrep-cap vectors exhibited a deletion within the rep gene. These results demonstrate that upon extended passage of the rAdAAVrep-cap chimeric vector, a portion of the rep gene is deleted.

Deletion of rep in the context of rAdAAVrep-cap is not site-specific, but does occur within rep

To determine the cause of deletions in the rep gene after the high passage number, the chimeric AdAAV virus was passed through 293 cells for 16 passages. PCR was car-

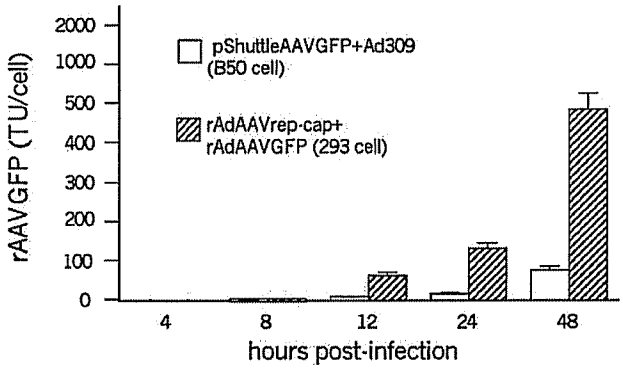

Figure 5 High-titer rAAVGFP production using two recombinant adenoviruses. The rAAVGFP was produced and titrated as described in the text. The open bar represents the titer of rAAV-GFP using plasmid DNA pshuttleAAVGFP transfection of B50 cells followed by Ad309 infection. The solid hatched bar represents the titer of rAAVGFP using two recombinant adenoviruses.

ried out and the full-length and smaller versions of AAV were cloned and sequenced. This indicated that the deletion within AAV always occurred within the rep sequence of rAdAAVrep-cap (Figure 6b), although the deletion was not site-specific in the two clones examined. The deletions that occurred in the rep gene after high passage of the rAdAAVrep-cap chimeric vector were sufficient to disrupt all the four rep genes of AAV. To demonstrate further if deletion of rep in rAdAAVrep-cap is correlated with AAV Cap protein expression, Western blot hybridization was carried out to detect AAV Cap protein expression. The results showed that high level of Cap proteins were detected in case of passage 4 (Figure 6c, lane 2), lower Cap proteins expressed at passage 8 (Figure 6c, lane 3), or no Cap proteins were detected in case of absent of rep gene expression at passage 16 (Figure 6c, lane 4). There was no Cap proteins detected in AdCMVLacZ infected 293 cells (Figure 6c, lane 1).

Discussion

The present results are the first to demonstrate the AAV rep and cap can be combined in the Ad E1-deleted genome, and that this hybrid rAdAAVrep-cap supports high-titer production of recombinant AAV when co-infected with AdAAV bearing the AAV ITRs, such as rAdAAVGFP. The failure of previous investigators to produce chimeric AdAAV could be due to the inhibition of expression of cellular factor(s) that mediate homologous recombination *in vivo*. The low titer production of rAdAAVrep-cap is likely due to AAV rep inhibition of Ad replication. The AAV rep inhibition of Ad replication has been reported before,[6,13] and our results further demonstrated that AAV rep also cause Ad instability. This inhibition was also observed in initial generation of rAdAAVrep-cap virus in 293 cells. The slow growth of rAdAAVrep-cap might account for the previous failure to observe production of this vector after previous attempts to identify this recombinant by *in vivo* recombination. Our speculation is that because wild-type Ad is produced faster than recombinant AdAAV, subsequent infection of adjacent cells including those cells producing recombinant AdAAV could be destroyed limiting production of mature AdAAV.

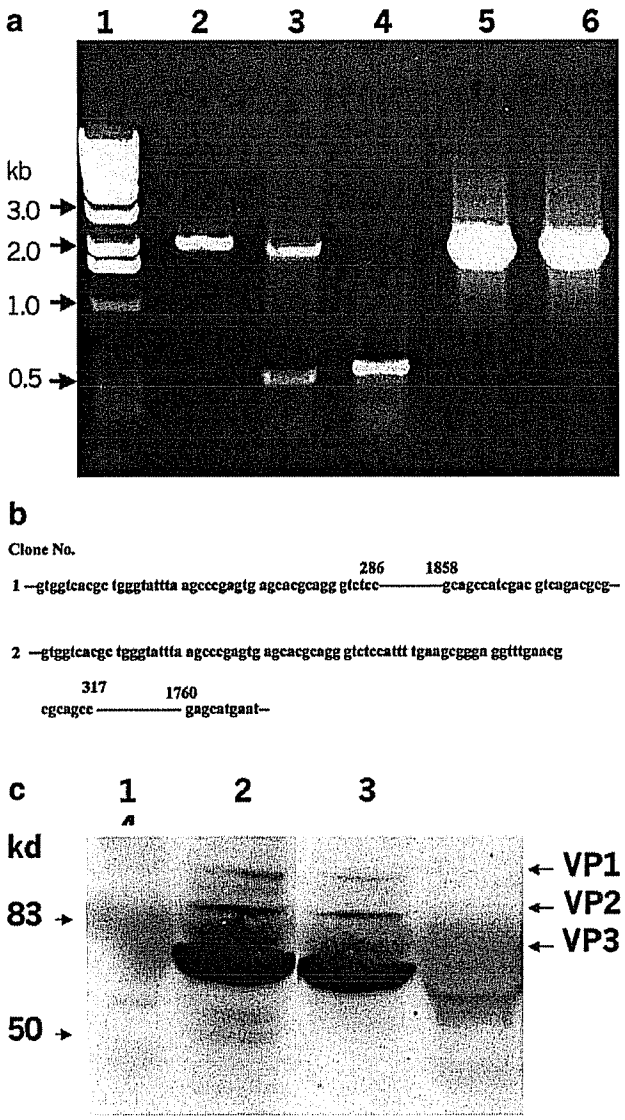

Figure 6 Non-homologous deletion of AAV rep gene after short-term passage of rAdAAVrep-cap. rAdAAVrep-cap was passed in 293 cells, and the DNA was isolated using the Hirt extraction procedure, or total protein was extracted as described in Materials and methods and used for Western blot of AAV cap proteins. The rep gene was amplified by PCR. (a) Lane 1, 1 kb DNA; lane 2, four passages; lane 3, eight passages; lane 4, 16 passages; lane 5, pSub201 wild-type AAV plasmid DNA as a control; lane 6, pAdAAVrep-cap as a template as a control. (b) Sequence analysis of rep gene deletion in rAdAAVrep-cap. The rep PCR products were cloned in p2.1PCR vector and sequenced. The numbers above the AAV2 DNA sequence are based on Gene Bank sequence number M12469 for AAV2. (c) 50 μg of total proteins extracted from each sample treated as indicated in Figure 6c were loaded on 10% SDS-PAGE gel, and was blotted with anti-AAV capsid protein (B1 clone, American Research Production) and signal was detected using ECL system. Lane 1, protein extracted from 293 cells infected with AdCMVLacZ; lane 2, protein extracted from 293 cells transfected with rAdAAVrep-cap passage 4 (10 p.f.u./cell); lane 3, protein extracted from 293 cells transfected with rAdAAVrep-cap passage 8 (10 p.f.u./cell); lane 4, protein extracted from 293 cells transfected with rAdAAVrep-cap passage 16 (10 p.f.u./cell).

Although the production of relatively high titer of rAAV has been pursued extensively, it can only be achieved currently through transfection of multiple DNAs,[9] and it is difficult to undertake large-scale production at low cost. High-titer production of rAAV using two recombinant adenoviruses would enable large-scale production of rAAV.

Long-term passage resulted in rAdAAVrep-cap vectors that contained a non-homologous deletion of the rep gene. Previous investigators have proposed that expression of Rep can inhibit Ad replication by several mechanisms including altered cellular transcription at replication center formation.[13] Other investigators have suggested that Rep interferes with cell cycle factors, including ras, fos, jun, and cyclin A.[19–24] Other reports indicate that Rep might promote apoptosis by blocking Ad inhibition of p53.[25,26] Our results are consistent with a previous report that it is possible to generate an AdAAVRep in which there was low expression of rep78 under the control of the bacterial phage T7 promoter.[27] We propose that early expression of Rep is compatible with rAdAAVrep-cap replication, but that long-term or high expression of the Rep gene product confers a growth disadvantage on cells that contain an unmutated rAdAAVrep-cap, and that these cells either exhibit limited growth or apoptosis. This leads to survival and selective growth of cells containing rAdAAVrep-cap with non-homologous deletion of rep genes after many passages. This should not affect the utility of rAdAAVrep-cap, but indicates that after many passages, it may be necessary to obtain frozen stock for virus propagation. These results indicate that rAdAAVrep-cap and rAdAAVGFP can be used for high titer production of rAAV, but high passage number should be avoided by maintaining several aliquots of stock virus at low passage number.

Materials and methods

Cell culture, plasmid DNAs and restriction enzymes

293 Cells were purchased from Microbix, Inc. (Toronto, Ontario, Canada), and cultured in F12/DMEM media (Gibco, BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum. The B50 cell line expressing AAV Reps and Cap proteins was provided by Dr J Wilson (Institute for Human Gene Therapy and Department of Molecular and Cellular Engineering, University of Pennsylvania and The Wistar Institute, Philadelphia, PA, USA) and maintained and used for production of rAAV as described.[10] Plasmid psub201 containing AAV rep and cap genes were provided by Dr J Samulski (Human Gene Therapy Center, University of North Carolina at Chapel Hill, NC, USA)[30] and used for construction of rAdAAV-rep-cap and rAAVGFP. Padeasy1 and pShuttle plasmid DNAs were provided by Dr B Vogelstein (Baltimore, MD, USA)[31] and used for construction of recombinant adenoviruses. *E. coli* BJ5183 bacteria was also obtained from Dr Bert Vogelstein (The Howard Hughes Medical Institute, Johns Hopkins Oncology Center, and the Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD, USA) and used for production of recombinant plasmids. All restriction enzymes were purchased from New England Biolab (Beverley, MA, USA).

Construction of hybrid rAdAAVrep-cap

The *Xba*I fragment encoding AAV rep and cap genes was excised from psub201, and inserted into the *Xba*I polylinker site of the pShuttle vector[31] that contains polylinker sequences flanked by adenoviral sequences. The region upstream of the polylinker contains the Ad5 nt 34 931–35 935, as well as the ITR and packaging signal sequences of Ad5 (nt 1 to 480). The downstream region of the polylinker contains Ad5 nt 3534–5790. The Ad5 E1 sequence, between nt 481 and 3533, has been deleted.[31] After linearization with *Pme*I, 1 μg of pShuttleAAV was mixed with pAdeasy1. Forty μl of BJ5183 cells were added and electroporation was performed in 2.0 mm cuvette at 2500 V, 200 ohms, and 25 μF in a BioRad gene pulser electroporator (Hercules, CA, USA).[31] The recombinant clones were selected by culturing on 10 cm petri dishes containing L-agar plus 50 μg/ml kanamycin. The production of the correct recombinant pAdAAVrep-cap was identified by restriction enzyme digestion. To generate recombinant rAdAAVrep-cap, the pAdAAVrep-cap was linearized with *Pac*I, followed by transfection of 293 cells using the LipofectAmine technique (Gibco, BRL). The viral plaques were collected for evaluation of expression of the rep and cap genes by restriction mapping and Northern blot assay.

Construction of hybrid rAdAAVGFP

PSub201 containing wild-type AAV was digested with *Pvu*II to isolated 4.6 kb fragment that contains wild-type AAV genes flanked by the AAV ITRs, and then inserted into Klenow filled in *Xba*I and *Sal*I digested pshuttle (named as pshuttleAAV). The pshuttle AAVGFP was then constructed by replacing the *Xba*I fragment encoding the AAV rep and cap genes with the GFP expression unit. The GFP expression unit was obtained from pIRES-EGFP (Clontech, Palo Alto, CA, USA) by *Nru*I and *Xho*I digestion, then Klenow filled in *Nru*I and *Xho*I GFP expression unit was used to replace *Xba*I fragment of pshuttleAAV (named as pshuttle AAVGFP). The insertion of AAVGFP into the E1-deleted locus was checked by partial sequence analysis of the flanking region and by restriction mapping.

Restriction enzyme analysis of rAdAAVrep-cap and rAdAAVGFP viral DNA

The rAdAAVrep-cap produced on transfection of 293 cells by pAdAAVrep-cap was verified by restriction enzyme analysis. The viral DNA was isolated from the 293 cells using the Hirt DNA extraction procedure.[32] Briefly, 293 cells were infected with rAdAAVrep-cap (10 p.f.u./cell) for 30 h at 37°C, and the viral DNA was isolated from the 293 cells using the Hirt DNA extraction procedure.[32] The rAdAAVrep-cap viral DNA was purified by phenol/chloroform extraction followed by ethanol precipitation and resuspended in Tris-EDTA buffer (TE) for restriction enzyme digestion. The viral DNA (20 μg) was digested with *Xba*I, *Sal*I, and *Eco*RI then electrophoresed on 0.7% agarose gels at 3 V/cm overnight. The ethidium bromide-stained gel was visualized under UV lamp and photographed. Also, recombinant rAdAAVGFP was analyzed in the same way as described for rAdAAVrep-cap.

PCR amplification and sequence analysis of the VrAdAAVrep-cap viral DNA

The sequence of the rAdAAVrep-cap viral DNA was verified by sequence analysis after PCR amplification. Briefly, the rAdAAVrep-cap viral DNA was purified as described above and used as a template for PCR amplification. The PCR reaction was carried out using a pair of primers binding to the upstream (Ad5 GeneBank No. M73260, starting nt 430 5'TTG-GCG-TTT-TAT-TAT-TAT-AGT-CAG-CT3') and the downstream (Ad5 GeneBank No. M73260, starting nt 3571 3'TGT-AGT-TTT-GTA-TCT-GTT-TT-GCA-GC) regions of the AAV rep and cap insert of the Ad E1-deleted sequence. The PCR amplified product was subsequently sequenced using an ABI sequencer (Foster, CA, USA).

Northern blot analysis of expression of rAdAAVrep-cap in 293 cells

293 Cells were infected with rAdAAVrep-cap at 10 p.f.u./cell for 24 h. The total RNA was then extracted from the virally infected 293 cells using TriZol solution according to the manufacturer's instructions (Gibco-BRL). A 10 μg aliquot of the total RNA was electrophoresed through a 1.0% denaturing agarose gel at 120 V for 3 h at 4°C. The RNA was then transferred to nylon membrane and the AAV rep and cap mRNAs were detected with α[^{32}P]-dCTP-labeled AAV DNA fragment encoding capsid (*Xho*I and *Xba*I fragment of AAV genomic DNA from pSub201).

Immunoblot analysis of the AAV capsid proteins expressed by rAdAAVrep-cap in 293 cells

For analysis of capsid protein expression, 293 cells were infected with rAdAAVrep-cap (10 p.f.u./cell) as described above. The cells were harvested at 24 h after infection and washed with phosphate-buffered saline. The protein was extracted from cell lysates in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxychlolate containing the proteinase inhibitors, 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml each of aprotinin, leupeptin, and pepstatin. The protein extract (50 μg) was fractionated on a 10% polyacrylamide-SDS gel, and transferred on to a nitrocellulose membrane. Capsid proteins were detected by incubation with an anti-AAV capsid monoclonal antibody (B1 clone, American Research Product Inc., Belmont, MA, USA) at 4°C overnight followed by a goat anti-mouse horseradish peroxidase-conjugated secondary antibody and a chemiluminescent detection system (KPL, Gaithersburg, MD, USA).

Quantitation of production of rAAVGFP on co-infection with rAdAAVrep-cap

The production of rAAVGFP following co-infection of rAdAAVrep-cap and rAdAAVGFP into 293 cells was determined. The effectiveness of this strategy was compared with the transfection of pShuttleAAVGFP into B50 cells expressing AAV rep and caps using the calcium phosphate procedure followed by Ad309 infection (10 p.f.u./cell).[7,23] The rAAVGFP produced was purified from cell lysates by two cycles of $CsCl_2$ gradients followed by heat inactivation of recombinant adenoviruses at 56°C for 30 min as described.[9,10] To obtain an accurate titer for the rAAV-GFP if was necessary to eliminate possible contamination with rAdAAV-GFP, which also would result in expression of the GFP protein in the test cells. A neutralizing adenoviral antibody 1D6.14[9] was first titrated by limited dilution with a constant amount of AdCMVLacZ on 293 cells. Briefly, 1D6.14 antibody was 10-fold diluted in PBS, then mixed with equal volume of AdLacZ (1×10^9 p.f.u.). After incubation 37°C for 1 h, the mixture was added on to 80% confluency of 293 cells, and incubated at 37°C for 3 days. The titer of neutralizing antibody was determined based on 100% blocking infectivity of 1×10^9 p.f.u. AdCMVLacZ virus. Therefore, the $CsCl_2$-purified and heat inactivated rAAV-GFP was incubated with the predetermined 100% neutralizing adenoviral neutralization antibody, 1D6.14,[9] at 37°C for 1 h, before infection of 293 cells with a 10-fold serial dilution. Then rAAVGFP was inoculated on to 24-well plates of 293 cells. After 2 h incubation at 37°C in a CO_2 incubator, the cells were washed with PBS, and incubated for an additional 48 h at 37°C. The GFP-positive cells were then counted using fluorescent light microscopy and the titer in transduction units (TU) per cell was determined as the number of GFP positive cells × dilution factor × (volume of initial viral inoculation/total number of initially seeded 293 cells).

The level of contaminated either rAdAAVrep-cap or rAdAAVGFP was quantified by a plaque assay of rAAV-infected 293 cells. The level of contamination was less than 1 p.f.u. of both virues per 1×10^{10} TU of rAAVGFP.

The absence of significant wt AAV contamination was confirmed by immunocytochemical staining of 293 cells that were co-infected with rAAVGFP and Ad309. As a positive control, 293 cells were also transduced with psub201 wild-type AAV plasmid DNA followed by Ad309 infection of 293 cells followed by incubation for 48 h. The 293 cells were then fixed in 2% formaldehyde + PBS. The expression of the AAV Rep protein in the 293 cells was determined by immunocytochemical staining using an anti-Rep monoclonal antibody (Clone 303.9, American Research Products Inc., Belmont, MA, USA). The levels of contamination with wild-type AAV-2 were less than one functional particle per 1×10^{10} TU of rAAVGFP.

Southern blot detection of recombinant AAVGFP replication

Efficient replication of recombinant AAVGFP was determined by the hybridization of a ^{32}P-dCTP labeled GFP probe to a blot of rAAVGFP DNA prepared using the Hirt extracted method.[34] Briefly, 293 cells were infected with $CsCl_2$-purified rAdAAVGFP recombinant virus at an MOI of 10 p.f.u./cell with or without rAdAAVrep-cap helper virus. At 24 h or 40 h after the infection, the cell pellets from 10-cm dish was resuspended in 500 μl of 20 mM Tris-HCl-20 mM EDTA (pH 8.0) and lysed by addition of 30 μl of 10% sodium dodecyl sulfate (SDS). The cell lysate was incubated at 37°C for 1 h with 100 μg/ml of proteinase K and brought to a final concentration of 800 mM NaCl. After incubation on ice for 2 h, the cell lysate was centrifuged at 16000 *g* at 4°C for 30 min. The supernatant was collected and subsequently extracted with phenol and chloroform. The low molecular weight DNA was precipitated with ethanol, rinsed with 70% ethanol. The samples were resuspended in 100 μl of TE buffer (pH 7.5) containing 100 μg/ml of DNAase-free RNAse and digested with *Dpn*I for 2 h. The DNAs were separated on a 1% agarose gel, followed by blotting on to a nylon membrane. The South-

ern analysis was performed with a GFP DNA probe (0.7 kb *Not*I fragment of GFP from Green GFP, BRL), that had been labeled with ^{32}P-dCTP using a random primer kit (Amersham, Piscataway, NJ, USA).

Characterization of rAdAAVrep-cap production

To evaluate the activity of the AAV rep gene in the context of rAdAAVrep-cap, 293 cells (1×10^5) were cultured in a 24-well plate then infected with either rAdAAVrep-cap or AdCMVLacZ constructed as described[33] at 10 p.f.u./cell for 1 h, followed by washing to remove unattached virus. The infected cells were harvested daily until 95% of the cells developed visible cytopathic effects (CPE). The cells were then harvested and the virus released by subjecting the cells to three freeze–thaw cycles. The viral titer, expressed as p.f.u./cell, was titrated using a standard plaque assay on 293 cells, and calculated as the number of plaques × dilution factor × (volume of initial viral inoculation/total number of initial seeded cells).

PCR analysis of rep deletion

The rAdAAVrep-cap chimeric vector was passed through 293 cells for four passages, eight passages and 16 passages. The rAdAAVrep-cap DNA was isolated after each passage using the Hirt extraction procedure.[34] The rep gene was amplified by PCR using the rAdAAVrep-cap genomic DNA as a template. One of PCR primer (P1) sequence (5′-starting nt 430 of Ad5 5′TTG-GCG-TTT-TAT-TAT-TAT-AGT-CAG-CT3′) was designed to adenovirus vector DNA that is adjacent to the upstream region of the P5 promoter of AAV, and the other PCR primer (P2) sequence (5′-AGA-GAG-AGT-GTC-CTC-GAG-CCA-3′) was designed to recognize the incorporated AAV rep (Figure 1). Thirty-four cycles of PCR amplification were performed in a 50-μl reaction volume containing 1 × reaction buffer (1.5 mM of $MgCl_2$, 200 μM dNTPs, 50 pmols of each primer, and 2.5 U of Taq DNA polymerase) (Promega, Madison, WI, USA) using a Perkin-Elmer Gene Amp PCR System 9600 (Norwalk, CT, USA). Each cycle consisted of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 3 min. The amplified PCR products were directly sequenced using the fluorescent dye method.

Statistical analysis

Results were expressed as mean ± standard error of the mean (s.e.m.). The two-tailed Student's *t* test was used for statistical analysis. A *P* value of less than 0.05 was considered statistically significant.

Acknowledgements

We thank Dr James Wilson for providing B50 cell line, Dr J Samulski for plasmid psub201, Ad309 and Dr Bert Vogelstein for plasmid DNAs pshuttle, pAdeasy1. We also thank Mr M Spell and Dr T Rogers at the FACS Core Facility at UAB for helping to do the FACS analyses. We also thank M Linda Flurry for excellent secretarial work and Dr Fiona Hunter for editorial assistance. This work is supported by NIH grants R01 AG 11653, RO1-AI-42900, N01 AR 6–2224, and CA20468, and a Birmingham VAMC Merit Review Grant. Huang-Ge Zhang is a recipient of an Investigation award from American Arthritis Foundation, and Hui-Chen Hsu is a recipient of a postdoctoral fellowship from Arthritis Foundation.

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